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14. ABSTRACT
Learning difficulties and attention deficits are observed in 40-50% of children with Neurofibromatosis Type 1 (NF1). Additionally, many NF1 patients exhibit specific deficits in visuo-spatial tasks, such as the Judgment of Line Orientation task. Spatial learning is also disrupted in heterozygous mouse Nf1 mutants, and can be rescued by reducing Ras activity either genetically or pharmacologically. Conversely, olfactory learning defects observed in Nf1 mutant fruit flies are cAMP-dependent rather than Ras-dependent. Visuo-spatial learning in flies depends on the Central Complex region of the brain, which is distinct from the Mushroom Body region that is essential for olfactory learning. We hypothesize that visual learning defects in Nf1 mutant flies will be Ras dependent, and that this fruit fly model can be exploited to assay potential therapeutic treatments for NF1 cognitive deficits. We propose to assay visual learning in Nf1 mutant flies, and in flies where Ras activity is disrupted in specific regions of the adult brain. This report describes the generation of transgenic fly lines to localize RNAi knockout of NF1, Ras and MAPK, and to express RasGAP defective NF1 isoforms, using the Gal4/Gal80ts/UAS (TARGET) system for precise temporal and spatial control. We have also assayed olfactory learning acuity in Nf1 mutant flies that have been fed drugs that reduce Ras activity, or affect downstream targets of Ras/NF1, such as farnesyl transferase inhibitors, statins or rapamycin. Construction of the visual learning apparatus is in progress.

15. SUBJECT TERMS
Drosophila, NF1, Ras, AC, visual learning, pharmacology, transgenic flies, RNAi

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INTRODUCTION:

Learning difficulties and attention deficits are observed in 40-50% of children with Neurofibromatosis Type 1 (NF1). Additionally, many NF1 patients exhibit specific deficits in visuo-spatial tasks, such as the Judgment of Line Orientation task. Spatial learning is also disrupted in heterozygous mouse *Nf1* mutants, and can be rescued by reducing Ras activity either genetically or pharmacologically. Conversely, olfactory learning defects observed in *Nf1* mutant fruit flies are cAMP-dependent rather than Ras-dependent. Visuo-spatial learning in flies depends on the Central Complex region of the brain, which is distinct from the Mushroom Body region that is essential for olfactory learning. We hypothesize that visual learning defects in *Nf1* mutant flies will be Ras dependent, and that this fruit fly model can be exploited to assay potential therapeutic treatments for NF1 cognitive deficits. We propose to assay visual learning in *Nf1* mutant flies, and in flies where Ras activity is disrupted in specific regions of the adult brain. We will use the Gal4/Gal80ts/UAS (TARGET) system to localize RNAi knockout of NF1, Ras and MAPK, or to express RasGAP defective NF1 isoforms with precise temporal and spatial control. These flies will also be assayed for MAPK and AC activity. In addition, we will assay visual learning acuity in *Nf1* mutant flies that have been fed drugs that reduce Ras activity, or affect downstream targets of Ras/NF1, such as farnesyl transferase inhibitors, statins or rapamycin.

BODY:

During this reporting period (June 2007 – June 2008) this research resulted in the generation of transgenic fly lines for RNAi knockout of NF1, and other Ras pathway genes, and for expression of mutant NF1 proteins. Pharmacologic testing of flies was performed using lovastatin, including assays of survival, climbing ability and olfactory learning. We were granted a 6 month no-cost extension to finish construction of the visual-spatial learning apparatus and testing of *Nf1* mutant flies.

Task 1. Assay *Nf1* mutant flies for visual learning defects.

In order to assay visual learning in flies, we have designed and built an apparatus that incorporates a wing-beat analyzer (WBA), in place of the torque meter used to gauge the fly's direction of flight by the Heisenberg lab (Wolf & Heisenberg, 1992). The WBA has been used extensively for analysis of Drosophila flight dynamics in the Dickinson lab (Fry et al., 2008), and offers more precise capture of the flies' motion than the torque meter. We have retained Heisenberg's rotating panorama for presentation of visual cues, since it provides better control of the visual field and is less prone to overheating the flight arena than the LED display field used by Dickinson. Successful implementation of this modified apparatus will facilitate research, both by our lab and others, in the field of visual learning.

The WBA is a custom built piece of equipment, which led to some delays in its procurement, since we had to wait until other WBA orders were filled. Consequent delays

in construction of the flight arena, and writing and testing of software to run the apparatus were unavoidable. This task has not yet been completed but we expect to finish testing the apparatus and begin testing *Nf1* null mutants shortly. We have obtained fly lines with mutations that are known to affect visual learning including *rutabaga* and *ignorant* flies (Putz et al., 2004; Liu et al., 2006), to use as controls to test the visual learning apparatus.

***Task 2*. Disrupt NF1 expression in subregions of the adult fly brain to ascertain the anatomical location of visual learning deficits.**

We have obtained an NF1-RNAi transgenic Drosophila line that can be used to knockout expression of NF1 in specific tissues or subregions of the brain, using the UAS-Gal4 system to control expression of a double stranded NF1 RNA fragment. This transgenic line was part of a collection of RNAi lines that covers the entire Drosophila genome (Dietzl et al., 2007) which is available from the Vienna Drosophila Resource Center (VDRC). The NF1-RNAi construct (VDRC #13892) is directed against sequences found in all NF1 isoforms (Table 1), however, the transgene insertion in this particular line disrupts a vital gene, causing homozygous lethality, which limits its usefulness. Accordingly, we have used genetic crosses with a line carrying the delta23 transposase to move the transgene to other locations in the genome and establish new lines. We were unable to precisely locate the original transgene insertion site by inverse PCR, however, we have utilized conventional genetic mapping to identify several new lines where the transgene has moved to another chromosome. These lines are no longer lethal, indicating precise removal of the transgene and restoration of the essential gene that was interrupted. Because of all the genetic manipulations that were necessary, it has taken much longer than we expected to get the RNAi knockout of NF1 up and running.

In addition to the problems of lethality with the NF1-VDRC line, it also has a potential off-target effect due to a small region of sequence similarity with the Neurotactin gene. We have therefore made three additional UAS-RNAi constructs to knockout expression of NF1 (Table 1). One of these targets a region in the middle of NF1 that is common to all known isoforms, while the other two constructs are directed against NF1 isoforms with different COOH terminal domains. We also utilized a new vector, pValium, that only recently became available (Ni et al., 2008). This vector facilitates cloning of the NF1 PCR fragments in a head-to-head, or tail-to-tail, orientation in order to produce double stranded NF1 RNA when expressed in flies under control of the Gal4-UAS system. This vector further allows us to insert the transgenic constructs in defined locations in the fly genome to avoid any effect of transgene insertion site on expression of the constructs. This greatly reduces the time needed to make and screen transgenic lines, and also stops insertional lethality issues. These constructs have been verified by PCR and sequencing, and injected into flies to generate transgenic lines.

We have several transgenic fly lines that express Gal4 in the whole brain or mushroom bodies (elav, armadillo, scabrous, MB247, 201y, OK107 and c747). We have also obtained a number of lines that express in the central complex region that is required for visual learning, including the fan-shaped body (107y, 210y, c5 and c205) and the ellipsoid body (7y, 52y and c232). In addition, we now have several lines for the

expression of Gal80ts under control of the tubulin promoter (McGuire et al., 2003), as well as some recently published Gal4ts lines (Mondal et al., 2007).

Table 1: Anti-NF1 DS-RNAi constructs

Construct (VDRC#)	Primer 1	Primer 2	Fragment Size (bp)	Isoform(s)Targeted
Nf1 (#13892)	CGCGAATTCCCATTCTGCCAC TCGCCCTTC	GCGTCTAGAGACCCACTTTGA TAGCCACCTTTGT	380	Nf1-RB, Nf1-RC, Nf1-RD
Nf1-N	GTCTAGACGAGGAGATGTCCA CCCAAC	AGAATTCTCATCATTACTTCC ACAAGC	349	Nf1-RB, Nf1-RC, Nf1-RD
Nf1-NB	AGAATTCCATTCTCATCGGAA TCATTAG	GTCTAGAATCGTTTGGAGTGT TGGGAC	300	Nf1-RB
Nf1-ND	GTCTAGACCACTGAATGCGAA AGAAGG	AGAATTCATGTGCAACATGCC GTCTTC	349	Nf1-RD

All of the obtained RNAi and Gal4 lines have been outcrossed for 5 generations to our standard behavioral control line in order to remove any effect of background mutations that can affect behavior. The fly lines have been remade into homozygous state, and are now being tested for effects on survival, olfaction and shock reactivity. Once these important control experiments are finished we will test the olfactory learning ability of the RNAi lines in the absence of a Gal4 driver to check that insertion of the RNAi construct does not affect learning. Then we can assay the effect on learning when the RNAis are expressed in the brain, particularly the mushroom bodies. We anticipate that the visual learning apparatus will then be ready for us to begin testing these lines for visual learning defects, using Gal4 drivers that express in the central complex region of the brain.

***Task 3*. Use localized RNAi knockout or expression of mutated NF1 proteins to assay the Ras/NF1-dependence of visual learning:**

In addition to the NF1-RNAi lines described above, we have obtained two Ras-RNAi lines, two MAPK-RNAi lines, an EGFR-RNAi line, three TSC-RNAi lines and an S6K-RNAi line from the VDRC (Dietzl et al., 2007), as shown in Table 2. Some of the VDRC lines are lethal, and all are inserted randomly into the genome. This could potentially affect expression levels of the RNAi constructs, or interfere with other genes that are important for Drosophila behavior. The lines have been outcrossed and rehomozygosed, and are being tested for survival and sensory deficits, prior to assays of olfactory or visual learning ability, with or without Gal4 controlled transgene expression in specific brain regions.

We have made additional UAS-RNAi constructs to knockout expression of the TSC1 and TSC2 genes (Table 2), in order to take advantage of the new pValium vector system, and generate lines that are inserted in a defined location to avoid insertion site effects. These plasmids have been verified by PCR and sequencing and injected into flies to generate transgenic lines. We are also making additional RNAi lines directed against Ras1, Ras2, MAPK, and S6K that will utilize the pValium vector system.

Table 2: Other Ras/MAPK Pathway DS-RNAi constructs

Construct (VDRC#)	Primer 1	Primer 2	Fragment Size (bp)	Isoform(s)Targeted
Ras1 (#12553)	CGCGAATTCACGGAATACAAA CTGGTCGTCGTTG	GCGTCTAGAGGGCACCTCTTC GGCATCCT	327	Ras85D-RA
Ras2 (#1517)	CGCGAATTCGCTTCGATGAGA TCCCCAAGTTCC	CGCTCTAGAGCAGCACTTCCT CTTGCCCTTCTT	349	Ras64B-RA
MAPK (#4697)	CGCGAATTCGGATCTACGGAA GTTCCTCAATCTAATGCT	CGCTCTAGAAAACATCTCTCA TTTGGTCTATGCTATCAACTC	301	rl-RA, rl-RB, rl-RC, rl-RD, rl-RE, rl-RF
EGFR (#1654)	CGCGAATTCGGAGCACGCAAA TCGCCAAG	CGCTCTAGAGCCAAAGGTCAG CAGTTCCCAAA	284	Egfr-RA, Egfr-RB
Tsc1 (#11836)	CGCGAATTCTTGAGGTGTTCG GGCATTTGG	GCGTCTAGAGGACGGGCAGCG ATAGGTTG	336	Tsc1-RA
Tsc2 (#1454)	CGCGAATTCTTACCACAAGCC CACCCGACA	CGCTCTAGAGGGTAAAACGAG GGCGTGGAA	252	Gig-RA
S6K (#6646)	CGCGAATTCTGGCAAAGGTGG TTATGGCAAAGT	GCGTCTAGACGGGCTTCAGAT CGCGGTAG	374	S6k-RA
Tsc1-T1	AGAATTCAGTCCGCATAACGA GATTGC	GTCTAGAGAGACATCCGGGAA ACTGAAC	316	Tsc1-RA
Tsc2-T2	AGAATTCCAAGGATCGTCTGC ATCACCTGC	GTCTAGAGAGCCTCGATCGTC TTAATGC	304	Gig-RA

Pre-existing lines that express GAP-defective human NF1 transgenes have been extensively tested for rescue of *Nf1* mutant body size and olfactory learning and memory defects, and biochemical assays have been performed (Hannan et al., 2006; Ho et al., 2007). We have also generated a number of additional human NF1 constructs that carry clinically identified missense mutations affecting regions of the NF1 protein outside the GAP domain, as well as both PKA and MAPK phosphorylation site mutants (Table 3).

Because the *Nf1* gene is on the third chromosome it is preferable to have fly lines with the human NF1 mutant transgene on the second chromosome, to facilitate creation of lines that carry the human transgene in the *Nf1-/-* mutant background. Lines without second chromosome insertions have been crossed to the delta23 transposase line to move the transgenes from their current insertion sites, and new lines are being mapped currently.

Transgenic flies carrying insertions of human NF1 mutants on the second chromosome have been crossed into the *Nf1-/-* mutant background. These lines are currently being tested for effects on mutant body size, and olfactory learning and memory defects, when the transgene is expressed in the nervous system under Gal4-UAS control.

Table 3. Human NF1 mutant lines.

Mutation	**Effect**	**2nd Chromosome Lines**
S818A	PKA site	F302, F303, F307, F310
991delM	Patient mutant	C604, C609
K1105R	Ubiquitin site	-
R1391S	GAP domain	E117a, E117b, E117c
1658delIY	Sec1p domain	-
L1932P	Patient mutant	T201, T205, T207
2366delNF	Patient mutant	-
S2739A	MAPK site	-

***Task 4*. Rescue visual learning defects pharmacologically with farnesyl transferase inhibitors, statins or rapamycin.**

We have optimized the drug delivery regimen using lovastatin, a statin that is commonly prescribed for cholesterol reduction, which has been shown to rescue spatial learning defects in *Nf1* mutant mice (Li et al., 2005). The effects of both chronic and acute exposure to drug were tested. For chronic exposure, adult flies were placed in bottles or vials containing fly food supplemented with lovastatin for several days prior to testing learning ability. Both potato and corn based fly food were tested. For acute exposure, flies were placed for 12-16 hours in vials containing tissue soaked with a solution of 4% sucrose plus lovastatin. Control flies were exposed to non-supplemented fly food or 4% sucrose alone. Uptake of drug was monitored by co-ingestion of colored food dye in both chronic and acute exposure paradigms.

We have assayed survival, sensory controls and olfactory learning in lovastatin treated flies. We have shown that survival of adult flies treated acutely with 0.1 mg/ml lovastatin is not affected for up to 3 days after drug treatment (Figure 1). We have also shown that there is no effect on survival of flies after chronic exposure to drug at concentrations up to 0.75 mg/ml (not shown).

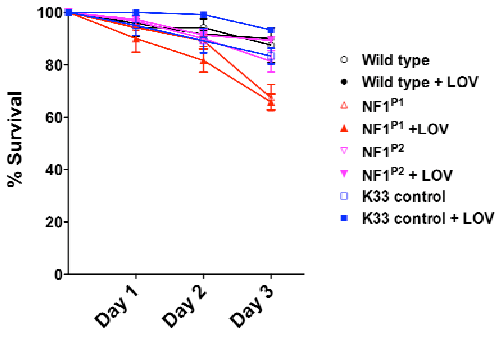

Figure 1. Adult Survival. Acute exposure to lovastatin (0.1 mg/ml; 18-24 h) does not affect survival in NF1mutant flies ($NF1^{P1}$, $NF1^{P2}$) or control flies (2202u, k33) up to 3 days post-drug exposure.

Behavioral testing was performed on flies exposed to lovastatin at a concentration of 0.1 mg/ml. Locomotor activity, shock reactivity and olfactory acuity were not affected by drug treatment (not shown). Significant but partial rescue of olfactory learning was observed in two different *Nf1* mutants ($NF1^{P1}$ and $NF1^{P2}$) comparing drug treated flies with non-treated controls, as shown below for acute exposure to drug (Figure 2). Learning was not affected by chronic or acute exposure to drug in normal control flies (22020u and K33 lines).

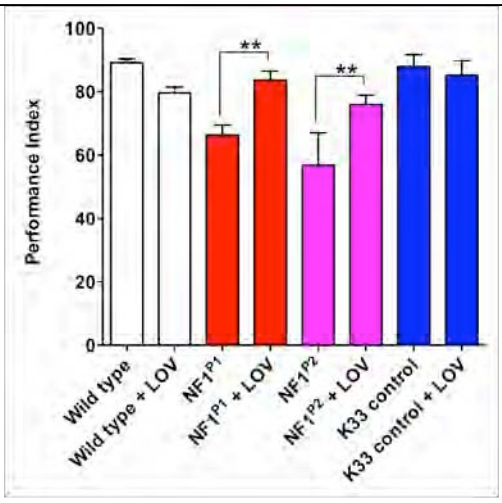

Figure 2. Adult Learning. Acute exposure to lovastatin (0.1 mg/ml; 18-24 h) rescues olfactory learning defects in NF1mutant flies ($NF1^{P1}$, $NF1^{P2}$) but has no effect on wild type or parental control flies (2202u, k33). (** $p<0.05$, Tukey-Kramer HSD; n = 15, 3, 9, 7, 5, 6, 7, 5)

We are currently repeating these assays using higher concentrations of lovastatin to see whether complete rescue of learning defects can be achieved in the *Nf1* mutants. For future experiments we will utilize acute exposure to drugs since this minimizes the amount of drug needed while still producing a measurable effect. Western

analysis of MAPK activity in lovastatin treated flies versus controls is currently underway.

We will also begin testing of survival, behavioral controls, and olfactory learning for two other drugs, rapamycin and rolipram, which are both approved for use in humans. Rapamycin is an immune suppressive, anti-proliferative drug that inhibits mTOR (mammalian target of rapamycin), a protein which is regulated by NF1 and TSC proteins, and may affect cognitive functions via the S6K pathway. Rolipram is a cAMP-specific phosphodiesterase (PDE4) inhibitor marketed for its anti-inflammatory and anti-psychotic effects. Inhibition of PDE4, which is normally responsible for decreasing cAMP concentrations, may facilitate learning. Levels of phospho-MAPK or phospho-S6K in treated versus control flies will be measured using Western blot analysis, while cAMP levels will be assayed using an ELISA based kit . We will begin assays of visual learning once we have finished modifications to the apparatus as described above.

KEY RESEARCH ACCOMPLISHMENTS:

- Design and construction of new visual learning apparatus.
- Generation of transgenic lines for RNAi knockout of NF1, EGFR, Ras, MAPK, TSC1, TSC2 and S6K.
- Generation of transgenic lines for expression of mutant NF1 proteins.
- Assays of survival and locomotor activity in *Nf1* mutant flies treated with lovastatin.
- Demonstration of rescue of olfactory learning deficits in *Nf1* mutant flies treated with lovastatin.

REPORTABLE OUTCOMES:

Manuscripts:

Shilyansky C., Li W., Legius E., **Hannan F.,** Wiltgen B., Hardt M., Krab L., Elgersma Y., Hunter-Schaedle K., Acosta M. & Silva A.J. (2008). Molecular and cellular mechanisms of learning disabilities: a focus on neurofibromatosis type I. In: Animal and Translational Models of Behavioral Disorders, Vol 2, R.A. McArthur & F. Borsini, eds. Elsevier, In press.

Meeting Abstracts:

Pharmacologic Rescue of Behavioral Deficits in Drosophila *Nf1* Mutants. Childrens Tumor Foundation National Neurofibromatosis Conference, Bonita Springs, FA, June 2008.

Presentations:

Pharmacologic Rescue of Behavioral Deficits in Drosophila *Nf1* Mutants. Childrens Tumor Foundation National Neurofibromatosis Conference, Bonita Springs, FA, June 2008.

Modeling Disease in the Fruit Fly Drosophila melanogaster. Club Neuron, New York Medical College, Valhalla, NY, November 2007.

Modeling Human Disease in the Fruit Fly Drosophila. New York Eye & Ear Infirmary Founders Meeting, Harvard Club, New York, NY, October 2007.

Funding Applied For:

Children's Tumor Foundation Drug Discovery Initiative

Children's Tumor Foundation Young Investigator Award

Cold Spring Harbor Drosophila Neuroscience Course

DoD CDMRP NFRP Concept Award

DoD CDMRP NFRP Exploration - Hypothesis Development Award

DoD CDMRP TSCRP Concept Award

Genetics Society of America DeLill Nasser Award

Howard Hughes Medical Institute Early Career Scientist Award

March of Dimes Research Program

National Institutes of Health Small Business Innovation Research R43/R44

Tuberous Sclerosis Alliance Innovator Award

CONCLUSIONS:

Research using the fruitfly Drosophila to study the function of the NF1 protein has provided remarkable insights for the NFI research field. Almost a decade ago, examination of fly *Nf1* mutants revealed a previously unsuspected role for NF1 in the regulation of AC activity (Guo et al., 1997). Subsequent studies have shown that this pathway is also affected in mice, and that human NF1 can control AC activity when it is expressed in flies. Expression of the human NF1 mutations and deletions in Drosophila has proven invaluable for defining the mechanism of NF1/Ras-dependent AC activation, and for definition of a functional domain outside the GRD of NF1 that controls NF1/Gsα-dependent AC activity and body size (Hannan et al., 2006). We have also been able to show that NF1 Ras-GAP activity is essential for protein synthesis dependent long-term memory in flies, while the C-terminal region contains sequences that are necessary for olfactory learning (Ho et al., 2007). This is the first time that a single protein has been shown to differentially affect these very distinct phases of memory formation. This underscores the crucial role of NF1 learning and memory.

We are uniquely placed to investigate the role of the AC and Ras pathways in the processes of learning and memory using our human NF1 mutants to disrupt the AC or Ras/MAPK pathways. We are also poised to examine the differential contribution of Ras versus AC pathways to both olfactory and visual learning, using the new technique of RNAi mediated knockout of genes in specific brain regions. This should also provide insight into the role of major neuron specific isoforms of NF1 in olfactory versus visual learning. It is critical that future creation of transgenic fly lines utilize vectors such as pValium, which allow insertion of constructs into defined locations in the fly genome, avoiding position effects on expression, and lethality of insertions, as well as allowing much more rapid generation and manipulation of transgenic lines.

Our demonstration that lovastatin can rescue olfactory learning deficits in *Nf1* mutant flies is the first to show that drugs affecting the NF1/Ras pathway in mammals can also be effective in fruit flies. These are exciting basic research results, of enormous general interest, which will stimulate further basic research into NF1 function in mammals, and generate new ideas for the development of therapeutic agents. This also opens up the tantalizing possibility of using our fruitfly system to screen for novel compounds that may be effective against symptoms of NF1 in humans, including cognitive deficits in children with NF1.

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